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The impact of species, respiration type, growth phase and genetic inventory on absolute metal content of intact bacterial cells

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Abstract

Metal ions are abundant in microbial proteins and have structural, catalytic or electron-transferring roles. Metalloproteins are especially prevalent in respiratory chains where they couple electron flow with proton translocation across the membrane. Here, we explore the hypothesis that anaerobic respiratory chains can be investigated by quantitative whole-cell metallomics of the key metals Fe, Co, Ni and Mo. Sensitive and strictly quantitative data were obtained by inductively-coupled plasma mass spectrometry when using a triple quadrupole instrument (ICP-QqQ-MS). Our experiments provide data on the absolute cellular metal content of *E. coli,* an enrichment culture of "*Ca*. Kuenenia stuttgartiensis", *Dehalococcoides mccartyi*, *Desulfovibrio vulgaris*, *Geobacter sulfurreducens* and *Geobacter metallireducens*. A major obstacle in whole-cell metallomics is the interference caused by metal precipitates, observed for *G. metallireducens* and *D. vulgaris*. In the other investigated organisms, whole-cell metallomics gave biologically meaningful information, e.g. high Fe and Co content in "*Ca. K. stuttgartiensis*" and higher Mo content in *E. coli* when grown under nitrate-reducing conditions. The content of all four metals was almost constant in *E. coli* from the late exponential phase allowing precise measurements independent of the exact duration of cultivation. Deletion or overexpression of genes involved in metal homeostasis (Ni transport or Mo-cofactor metabolism) was mirrored by dramatic changes in whole-cell metal content. Deletion of genes encoding abundant metalloproteins or heterologous overexpression of metalloproteins was also reflected in the whole-cell metal content. Our study provides a reference point for absolute microbial metallomics and paves the way for the development of fast and easy mutation screens.

Significance to metallomics

Metallomics looks at the broad and complex roles metals play in biology. Here, we determine absolute numbers of the transition metals Fe, Co, Ni and Mo in bacteria that use different anaerobic respiratory growth modes. The study shows in quantitative terms that different bacterial genera have different overall metal contents. We also show that whole-cell metallomics can provide information about the growth phase, the respiratory mode and the gene inventory of a cell.

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Introduction

Transition metals are abundant in microbial proteins[1, 2] with intracellular concentrations of combined free and bound species spanning the pico- to micromolar range.[3] They play roles in the stabilization of the three-dimensional protein structure, in electron transfer within proteins or in catalysis.[4, 5] In large respiratory multisubunit protein complexes metal ions are highly organized to form electrically conductive 'wires' through the proteins. Whereas the electron path through proteins is mostly formed by iron-sulfur clusters, the electron entry and exit points are often formed by specialized metal clusters[6, 7] characterized by the presence of transition metal cations with an incomplete *d*-shell, and which occur in two or more redox-states, such as Fe, Co, Ni, Cu or Mo. In biological systems cysteine residues of the protein backbone often contribute to the coordination of Fe and Ni to form redox-active FeS or NiFeS clusters[8]. Other redox-active metal centers are formed by metal-containing organic cofactors such as heme, cobalamin, cofactor F_{430}, and molybdopterin for the metals Fe, Co, Ni and Mo, respectively. These metal-containing cofactors have key functions, for example in cytochromes, organohalide reductive dehalogenases, archaeal methyl-CoM reductase and nitrate reductase, all enzymes involved in anaerobic electron transfer processes. Copper, in contrast, is best known for its involvement in cytochrome oxidase (complex IV) of mitochondrial, bacterial and archaeal respiratory chains. It functions together with heme cofactors in the final oxidation of cytochrome with oxygen,[9] and therefore is more involved in aerobic respiration. The same is true for the copper-containing quinoprotein amine oxidase from *E. coli*.[10] Copper metalloproteins are hypothesized to have appeared late in evolution as an adaptation to oxygen accumulation in the atmosphere.[11]

In anaerobic respiratory chains iron plays a more specific function e.g. in the formation of nickel-iron clusters in [NiFe] hydrogenases or in the active center of iron-only hydrogenases.[12] In the exceptional organisms *Ferroplasma acidiphilum*, iron is found in up to 86% of all cellular proteins.[13] Cobalt has been detected in many methyltransferases and mutases[14] and plays a major role in the terminal reductases in organohalide respiration. Nickel is crucial in many hydrogenases,[8, 15] urease, carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS). Prokaryotic formate dehydrogenases contain molybdenum complexed as molybdenum *bis* molybdopterin guanine dinucleotide (Mo-*bis*-MGD).[6] Molybdenum is also involved in terminal reductases of respiratory chains including nitrate, selenate, thiosulfate, polysulfide, tetrathionate, dimethylsulfoxide (DMSO), and trimethylamine oxide (TMAO) reductases.[16]

Metal homeostasis within microbial cells is maintained by a large number of different proteins with various functions including low- and high-affinity binding, active or passive transport across the cell membrane, storage, incorporation into metal clusters and redox-conversions and several studies on whole-cell metallomics have been published.[17-19] Over the last decades comprehensive analyses have

been done to identify deletion mutants impaired in specific parts of metal homeostasis and the functions of many proteins have been reported. However, the detection of such mutants is laborious and relies on biochemical and/or physiological tests. The heterologous overexpression of metalloproteins represents a major experimental challenge and has only recently been resolved, for example, for the [NiFe] hydrogenase[20-24] and cobalt-containing reductive dehalogenases[25-27] but metal incorporation has to be tested separately. In this respect, development of a complementary method to assess the overall metal content of cells, and giving an indication of the consequences of gene deletions or overexpression would be highly beneficial. A prerequisite for such an approach is that the deletion mutant affects strongly the overall metal content of a cell or that a heterologously expressed metalloprotein is present in large amounts to induce significant changes in the total cellular metal content.

Here, we tested the hypothesis that the growth state of an organism, its respiration mode and its genomic complement can be traced via whole-cell metallomics. We focused on *E. coli* as a model organism to study the metal content of Fe, Co, Ni and Mo in different growth phases, under different respiration regimes and in several mutants impacting metal homeostasis. We then compared the *E. coli* results with those obtained for microorganisms with alternative anaerobic metabolic modes, i.e. anaerobic ammonium oxidation (anammox), organohalide respiration, metal respiration, nitrate respiration and sulfate respiration. Anaerobic ammonium oxidation is a process in which ammonium and nitrite are used as electron donor and electron acceptor, respectively, in an anaerobic respiration and is catalyzed by specialized organisms. Here we used an enrichment of the anammox bacterium *Candidatus* Kuenenia stuttgartiensis which we cultivate in planktonic form in our laboratory.[28] Anammox bacteria are known to contain a high number of heme proteins.[29] In organohalide, metal, nitrate, fumarate and sulfate respiration halogenated organic compounds, metals, nitrate, fumarate or sulfate ions are used as the terminal electron acceptor in an anaerobic respiration while hydrogen or other reduced compounds are used as electron donor. In our study we used *Dehalococcoides mccartyi* strain CBDB1 as organohalide-respiring bacteria, *Geobacter metallireducens* as metal or nitrate-respiring bacteria, *Geobacter sulfurreducens* as fumarate-reducing bacteria and *Desulfovibrio vulgaris* as sulfate-reducing bacteria. All measurements were related to the number of cells, allowing us to obtain a general parameter for each cell type, which included both metals bound to proteins and metals not bound to proteins, present either as free ions or as precipitates within the cell.

Material and Methods

Chemicals and strain collection

All aqueous dilutions, cultivation media and ICP-MS solutions were prepared with ultrapure water (Millipore, Darmstadt, Germany). Nitric acid (65%, Suprapur) was purchased from Merck (Darmstadt,

Germany). ICP multi-element standard solution XVI was obtained from Merck (Darmstadt, Germany) and the rhodium internal standard from Fluka. All the other necessary chemicals and solvents were purchased from Merck-Aldrich (Germany) in the highest available quality.

E. coli strains BW25113 (DSM-27469) and MC4100 (DSM-6574), *Desulfovibrio vulgaris* strain Hildenborough number (DSM-644), *Geobacter sulfurreducens* (DSM-12127) and *Geobacter metallireducens* strain GS-15 (DSM-7210) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). "*Ca*. Kuenenia stuttgartiensis" culture WD[28] and *Dehalococcoides mccartyi* strain CBDB1[30] are routinely cultivated in our laboratory. *E. coli* mutant strains used in this study are described in Table 1.

Bacterial cultivation

Our trace element stock solution (1000×) contained 10 mL L^{-1} HCl (25% v/v), $FeCl_2 \cdot 4H_2O$ (7.55 mM), $CoCl_2 \cdot 6H_2O$ (0.79 mM), $MnCl_2 \cdot 4H_2O$ (0.5 mM), $ZnCl_2$ (0.51 mM), H_3BO_3 (0.1 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.15 mM), $NiCl_2 \cdot 6H_2O$ (0.11 mM), $CuCl_2 \cdot 2H_2O$ (0.1 mM). Se/W stock solution (1000×) contained $Na_2SeO_3.5H_2O$ (22.81 µM), $Na_2WO_4.2H_2O$ (24.25 µM) and NaOH (12.5 mM). Our vitamin-4 stock solution (1000×) contained D-biotin (5 mg L^{-1}), thiamine chloride-hydrochloride (50 mg L^{-1}), dicyanocobinamide (5 mg L^{-1}), dimethylbenzimidazole (5 mg L^{-1}). Luria Bertani Broth (LB medium) contained 0.1 g L^{-1} yeast extract, 0.1 g L^{-1} tryptone and 50 mg L^{-1} NaCl and was adjusted to pH 7.0 with HCl. The minimal medium contained 10 mM $(NH_4)_2SO_4$, 10 mM NaCl, 22 mM KH_2PO_4, 48 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 25 mM glucose, 1× trace element solution and 1× vitamin-4 solution. All anaerobic media were flushed with 20:80 N_2/CO_2 gas mix. The glucose stock solution and the vitamin-4 solution were filter-sterilized, and all other solutions were sterilized individually by autoclaving.

Aerobic cultivation of *E. coli* was done in 250 mL Erlenmeyer flasks with 100 mL of LB[31] or mineral medium, as described above, 37°C at 180 rpm on a rotary shaker. Anoxic cultivation of *E. coli* was done in nitrogen-gassed minimal medium (MM) with 20 mM KNO_3 as terminal electron acceptor in injection bottles closed with butyl septa and crimp caps. Samples of 1 mL were taken from *E. coli* cultures for metal analysis after 8, 10, 11, 13, 15, 17, 19, 21, 23, 33 and 48 h of incubation. Cells were harvested when the culture reached an OD_{600} of 1.0-1.2. When required, antibiotics were supplemented at the following final concentrations: ampicillin, 100 mg L^{-1}, chloramphenicol, 15 mg L^{-1}, and kanamycin, 25 mg L^{-1}.

Dehalococcoides mccartyi strain CBDB1 was grown in defined cysteine-reduced, bicarbonate-buffered, mineral medium with hydrogen as electron donor, 1,2,4,5 tetrabromobenzene as electron acceptor and 5 mM acetate as carbon source under strict anoxic conditions as described[32, 33]. The medium contained 1× vitamin-4 and 1× trace element solutions. Cultures were incubated statically under strict anoxic conditions at 30°C. Cultures attained cell numbers of approximately 10^8 cells mL^{-1}.

Desulfovibrio vulgaris strain Hildenborough was grown in defined anoxic liquid medium containing 1.5 mM K_2HPO_4, 5 mM NH_4Cl, 17.1 mM NaCl, 2 mM $MgCl_2$, 7 mM KCl, 1 mM $CaCl_2$, 1× vitamin-4 solution, 1× trace element solution, 1× Se/W solution, 4 mM cysteine as a reducing agent, Na-resazurin solution (0.1% w/v), 17 mM sodium lactate as electron donor and 11 mM K_2SO_4 as electron acceptor. The pH of the medium was adjusted to 7.2 with 1 N HCl. Cultures were grown statically at 30°C under strict anoxic conditions.

Geobacter sulfurreducens was cultivated in defined anoxic liquid medium (28 mM NH_4Cl, 4.2 mM Na_2HPO_4, 1.35 mM KCl, 30 mM $NaHCO_3$, 1× vitamin-4 solution, 1× trace element solution, 1× Se/W solution, 10 mM Na acetate as carbon source and electron donor and 50 mM sodium fumarate as electron acceptor. The cultures were incubated at 30°C under strict anoxic conditions.

Geobacter metallireducens was cultured at 30°C under strictly anoxic conditions in a mineral salt medium containing 30 mM acetate as carbon and electron source and 15 mM Fe(III)-citrate as electron acceptor as described previously.[34] *G. metallireduc*ens was also cultivated in a modified medium where 15 mM Fe(III)-citrate was replaced by 3 mM sodium nitrate and 1 mM Fe(III)-citrate. Cells were harvested when the culture medium became clear, indicating depletion of Fe^{3+}.

"*Ca*. Kuenenia stuttgartiensis" was cultivated in a highly enriched mixed culture with approximately 87% purity in a semi-continuous reactor as described.[28] The medium contained synthetic medium with 20 mM nitrite, and 20 mM ammonium, and 1× trace element solution but without organic carbon sources.[28] Cells were growing in a planktonic form and were harvested directly from the effluent of the reactor.

Harvesting and cell counting

A volume of 100–200 mL with a cell density of about 10^8 cells mL^{-1} was collected for *D. mccartyi, D. vulgaris, G. sulfurreducens* and *G. metallireducens* cultures and centrifuged under anoxic conditions at 6000 *g* and 16°C for 60 min. *E. coli* cultures were harvested by centrifugation at 6000 *g* and 16°C for 30 min. All cell pellets were washed twice with 10 mL PBS (phosphate buffered saline, pH 7.2; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and re-suspended in PBS for metal analysis. *G. metallireducens* and "*Ca*. Kuenenia stuttgartiensis" cells were harvested using Percoll silica particle beads (Pharmacia) as described in the manufacturer's instruction. Briefly, 6 ml of the Percoll suspension were mixed with 3 ml PBS and the mixture was centrifuged at 10,000 *g* for 30 min. About 20–40 ml of a culture with 5 × 10^7 cells mL^{-1} was concentrated to a final volume of 1 ml by centrifugation at 6000 *g* for 20 min. This suspension was gently applied onto the top of the gradient (total volume 10 mL) and the top half of the density gradient was gently mixed with the cell suspension (from 7 – 10 mL). This was followed by a centrifugation step for another 30 min at 6000 *g*. Iron particles settled to the pellet whereas *G. metallireducens* cells were distributed allover in the solution. The supernatant (around 8 mL) was diluted to 50 mL with PBS and centrifuged for 30 min at 6000 *g*. Around 45 mL of supernatant was discarded and the washing step was repeated again. For

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"*Ca*. Kuenenia stuttgartiensis", a bright red band appeared at the lower part of the falcon tube. The bright red band was collected separately and washed twice with PBS. Cell pellets were further re-suspended in PBS for metal analysis.

Cells were quantified by direct cell counting using epifluorescence microscopy as described earlier.[35] Briefly, 20 µL of cell suspension were incubated with 1.3 µL of SYBR Green I (Invitrogen, USA) (1:10000 dilution) in the dark at room temperature for 10 min. Eighteen µL of this suspension were added to an agarose-coated slide and immediately covered with a coverslip. Ten to fifteen images were taken with a camera mounted on the epifluorescence microscope and automatically counted via ImageJ and Microsoft Excel macros.[35] The sensitivity of this method is at about 10^6 cells mL^{-1} and the standard deviation around 10%.

Metal analysis

Cell suspensions of 500 µL were subjected to acidic digestion by adding 100 µL of concentrated (65% wt/vol) HNO_3 and incubated at 80°C in an ultrasonic water bath for 2 h. Samples were then diluted with ultrapure water to a final concentration of 2% of HNO_3. Rhodium internal standard solution was added to a final concentration of 1 µg L^{-1} to all samples. ICP multi-element standard solution Merck XVI was serially diluted in 2% HNO_3 to prepare calibration standards between 5 ng L^{-1} and 500 µg L^{-1} and also amended with the internal 1 µg L^{-1} rhodium standard. Samples were measured on a high resolution 8800 ICP-QqQ-MS (Agilent Technologies, USA) in direct infusion mode using an integrated auto-sampler at nebulizer speed of 0.3 rps (revolutions per second) and internal tube diameter of 1.02 mm for 45 s. The five metals Fe, Co, Ni, Mo and Rh were quantified. All measurements were performed in three technical replicates normalized with the internal standard and averaged by taking the mean value. All statistical analysis was performed using student t-test. We report our data as metal ions per cell because we can directly determine the amount of metal ions by ICP-MS and the microbial cell number by direct cell counting. When numbers of ions are reported it is meant to include the total amount of all chemical species of an element in a given sample including charged ions and uncharged atoms.

To reduce polyatomic interferences, especially from ArO and NaCl which have the same nominal mass of 56 as Fe and 60 as Ni, respectively, we used the Octopole Reaction System (ORS^3) with a collision/reaction cell (CRC). Hydrogen was added to the CRC at a flow rate of 3.0 mL min^{-1}. For all metals, the target masses of the first (Q1) and second (Q2) quadrupole were set to the same *m/z* value: Fe (56/56), Co (59/59), Ni (60/60), Mo (95/95) and Rh (103/103) with an integration time of 1 s under auto-detector mode. H_2 gas was introduced through the 2nd inlet line and argon gas (Ar) was added as a carrier gas via a dilution gas port located between the torch and the spray chamber. A Peltier-cooled (2°C) Scott-type spray chamber with a perfluoroalkoxy alkane (PFA) nebulizer was used as the injection system. All other parameters were optimized by the auto-tune function of the used ICP-MS

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MassHunter 4.2 workstation software. Further instrument operation parameters are given in Supplementary Table 1.

Results

Metal determination at nanomolar concentrations by ICP-MS

Calibration curves for the four target metals Fe, Co, Ni and Mo measured at 14 different concentrations from 5 ng L^{-1} to 500 µg L^{-1} in triplicate showed excellent linearity (R^2 = 0.99) for all four metals, confirming the applicability of the instrument. Such exact correlations with metal concentrations down to 20 pM for ^{60}Ni, 260 pM for ^{95}Mo and in the nanomolar range for ^{56}Fe and ^{59}Co (Table 2) were only achieved using hydrogen in the collision/reaction cell to reduce polyatomic interferences, but not using He as a collision gas or without gas. All further measurements were done with hydrogen in the reaction cell.

Metal analysis of whole cells

Relating ICP-MS measurements for Fe, Co, Ni, and Mo to the number of cells resulted in absolute numbers of metal ions per cell (Fig. 1). The absolute amount of Fe ions per untreated cell were between 6.78×10^5 for *D. mccartyi* and 1.08×10^{10} for *G. metallireducens.* Because we speculated that a large part of the Fe in *G. metallireducens* is enclosed in iron-containing abiotic particles we also analyzed *G. metallireducens* after centrifugation in a Percoll density gradient. This resulted in a number of 1.22×10^8 ions per cell, which were 2 orders of magnitude lower than without Percoll gradient washing. Nevertheless, the amounts of Fe ions per cell in *G. metallireducens* remained up to eight times higher than in "*Ca*. Kuenenia stuttgartiensis" (1.6×10^7) and in *G. metallireducens* grown under nitrate conditions (1.4×10^7), more than 30-fold higher than in *G. sulfurreducens* (3.9×10^6) and 12-fold higher than in aerobically grown *E. coli* in minimal medium (9.7×10^6). *E. coli* grown aerobically and anaerobically in mineral medium, together with *D. vulgaris*, had similar Fe contents of about 1×10^7 ions per cell. The highest amount of Ni ions per cell was observed in "*Ca*. Kuenenia stuttgartiensis" (1.6×10^5). Among all the analyzed bacteria, the amount of Co was maximal in *E. coli* grown aerobically (1.6×10^5) and anaerobically in minimal medium (2.3×10^5). The lowest amount of Co determined in the investigated bacterial species was observed with *E. coli* grown in LB medium. We observed the highest amount of Mo *D. vulgaris* (3.3×10^5 ions per cell) and the lowest in *E. coli* grown under aerobic conditions with LB medium (1.5×10^4) and in *G. metallireducens* grown with Fe(III) citrate as electron acceptor (1.8×10^4). *E. coli* grown under nitrate-reducing conditions in minimal medium contained 2.5-fold more Mo (5.0×10^4 ions per cell) than cells grown in the presence of oxygen (2.0×10^4 ions per cell).

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Metal content in different growth phases

To be able to correlate the metal content of a cell in batch culture to its physiology and the mode of respiration it uses, the metal content must be stable across all growth stages of a culture. To test if this was the case, we analyzed the content of all our four target metals in *E. coli* strain BW25113 in aerobic LB medium over a total incubation time of 50 h. As expected, the total concentration of metals bound in bacterial biomass increased with the incubation time (Fig. 2A-C), and this correlated with the increase in biomass (data not shown). When relating the biomass-bound metal concentrations to the cell numbers in the cultures the amounts of all four target metals per cell were essentially constant from 8 h of growth. This time point corresponded with the entry of the bacteria into the early stationary phase (Fig. 2D). Metal analysis from the exponential phase did not lead to consistent data.

E. coli mutant analysis

We then tested if metal analysis of whole cells can provide information about the genetic inventory of a cell. For this we focused on Ni and Mo and analyzed strains carrying two types of mutations: first, deletion mutants affected in their ability to transport or to incorporate specifically into proteins either Ni or Mo. This was achieved by impairing the whole Ni or Mo homeostasis (“systemic mutations”); and second, mutants lacking a specific nickel- or molybdenum-containing enzyme, which was achieved by deleting the gene encoding the corresponding Ni- or Mo-cofactor-containing subunit ("structural mutations"). In structural mutants only specific metalloproteins were deleted or overexpressed and we hypothesized that in such mutants the overall effect on metal-content should be lower than in systemic mutants.

E. coli strains with the systemic knockout mutations in *nikC* or *nikE*, both involved in Ni transport, or in *fnr*, a transcription factor required for expression of genes for both nickel transport as well as for hydrogenase,[36] contained significantly lower total amounts of Ni per cell than the *E. coli* wild-type BW25113 (Fig. 3A). Deletion of *moaA*, encoding a protein catalyzing the first step of molybdopterin biosynthesis, strongly reduced the total Mo content in *E. coli* cells compared to the wild-type. This was true for aerobic cultivation on two different media, but also under nitrate-reducing conditions and exclusion of oxygen (Fig. 3B).

A reduction of the Ni-content by about 33% in comparison to the wild-type was found for the mutant FTD147, which lacks the catalytic subunit of three [NiFe] hydrogenases in *E.coli* (Fig. 4A). In contrast, mutants of FTD147 in which the HybC, the large subunit of [NiFe] hydrogenase, was overproduced, was not reduced in Ni-content.

In wild-type *E. coli* MC4100 we observed about 2.5-fold higher Mo-content under nitrate-reducing conditions (5×10^4) than under aerobic conditions (2×10^4) (Fig. 4B). Deletions in genes for several subunits of the hydrogenase (Δ*hypB*, Δ*hypC*, Δ*hypE*), Δ*hyfB-R* and translation factor (Δ*selB*) resulted in a slightly lower Mo-content under aerobic conditions compared to the wild-type but in a strongly

reduced Mo-content under nitrate-reducing conditions compared to the wild-type. When we then heterologously overexpressed the *omeAB* genes from *Dehalococcoides mccartyi* strain CBDB1, hypothesized to encode a Mo-containing protein complex, we determined an increase in the mean level of the cellular Mo-content under both tested conditions. (Fig. 4B).

Discussion

The highly specific and sensitive detection of metal ions has been significantly improved within the last few years due to developments in mass spectrometric instrumentation and data processing. We demonstrate in this study that mass spectrometric detection using a triple quadrupole can be used for the quantification of low amounts of metals in complex biological samples. In our experiments, the best results were obtained by using the Octopole Reaction System as a hydrogen reduction cell to remove polyatomic interferences rather than using as a collision cell. This strongly reduced the background and resulted in high sensitivity and a strictly linear response.

We focused on the quantification of respiratory metals in intact cells to test the hypothesis that such data can give meaningful biological information on major processes within the cell in which the contributing proteins have either a strong systemic effect on cellular metal homeostasis or are abundant metalloenzymes. In microorganisms metal ions can be bound to organic molecules but can also be freely solvated in the cytoplasm or accumulating in abiotic precipitates within the cell or on its surface.[37, 38] The fraction of cellular metal ions bound to organic molecules is mainly bound to proteins, either directly to the amino acid chain or indirectly via a cofactor such as heme, cobalamin, cofactor F_{430} and NiFe-cofactor, or Mo-*bis*-MGD for Fe, Co, Ni and Mo, respectively. Abiotic precipitates appeared to be especially abundant in our Fe(III)-reducing model organism *G. metallireducens* grown with Fe(III) citrate as electron acceptor and our sulfate-reducing model organism *D. vulgaris* grown with sulfate as electron acceptor. Both species appear to form precipitates that greatly overshadow protein-bound metals. For *G. metallireducens* we could confirm this by washing Fe precipitates off the cell surface in a Percoll gradient, which revealed that protein-bound metals make up less than 1% of the total metal content. This was also confirmed by the results after cultivation of *G. metallireducens* with nitrate as electron acceptor, which prevented the formation of abiotic iron precipitates. In *D. vulgaris* this effect led to massive accumulation of Mo that could not be removed by washing of the cells. There is currently limited literature available describing the impact of intracellular Mo sulfides on the physiology of the cell or on the ecology of populations, as this should lead to general Mo depletion in sulfide-reducing environments. Taken together, the whole-cell metallomic data from iron- or sulfate-reducing bacteria must be interpreted with caution.

In contrast, the data from whole-cell metal analysis of bacteria that do not form metal sulfides can be meaningfully interpreted and can yield important biological insights. For example, the high amount of

Fe in "*Ca*. Kuenenia stuttgartiensis" indicates iron plays a crucial and dominant role in the anammoxosomes of these anammox bacteria.[39] The low Fe content of *D. mccartyi* strain CBDB1 is probably due to its low cell volume, which is reported to be only about 2% of that of *E. coli*.[40]

The highest Co content per cell was observed in *E. coli* grown in minimal medium. The Co-containing cofactor cobamide cannot be synthesized *de novo* by *E. coli*;[25] however, because the cobalamin precursor cobinamide was added to the mineral medium, cobalamin-containing enzymes could be nevertheless synthesized, e.g., the B_{12}-dependent methionine synthase, MetH. This enzyme might not be strongly expressed in LB medium as methionine is directly available to the bacteria. Co is also the central ion of cobalamin-dependent reductive dehalogenases involved in organohalide respiration in *D. mccartyi*.[40, 41] However, the absolute number of reductive dehalogenases estimated by mass spectrometric selective reaction monitoring was determined to be around 500 per cell[42] and therefore might not contribute much to the overall determined number of around 10,000 Co ions per cell as determined by ICP-QqQ-MS. This discrepancy needs to be further explored.

By far the highest Ni content was found in "*Ca*. Kuenenia stuttgartiensis". Genomes sequences are available for the highly enriched strains KUST and MBR1[43, 44] and indicate the coding capacity for a Ni transporter (CbiNLMQ), a Ni-containing CO-methylating acetyl-CoA synthase (CODH/ACS)[43] and one [NiFe] hydrogenase complex. The activity of CODH/ACS in anammox bacteria was biochemically demonstrated.[44] As it is involved in carbon fixation for autotrophic growth it is anticipated to contribute substantially to the high Ni content in *Kuenenia* species.

As indicated above, the high Mo content in *D. vulgaris* is likely the result of its precipitation as MoS_2,[38] although Mo transport[38] and the expression of Mo-cofactor-containing formate dehydrogenases[45, 46] has been described for *D. vulgaris*. The fact that Mo precipitates could not be separated from cells in a Percoll gradient could indicate the intracellular or periplasmic location of these precipitates or at least their tight association with the cells. Others have suggested that such $Mo(IV)S_2$ precipitates are formed after Mo(VI) to Mo(IV) reduction in the periplasm of sulfate-reducing bacteria.[38] With *E. coli* the comparison of the Mo content in cells grown in oxic or anoxic mineral medium with nitrate as electron acceptor resulted in the expected higher Mo content in anoxically grown cultures, presumably due to the expression of Mo-containing formate dehydrogenase and nitrate reductase, which are present in significant abundance in these cells.[47, 48] These data indicate that the metal content of *E. coli* accurately reflects the respiration mode used by the bacterium.

The monitoring of *E. coli* throughout its batch cultivation revealed that the content of all four investigated metals is remarkably stable from the late exponential phase onwards for over more than 40 hours of incubation. This stability allows a precise comparison of *E. coli* strains in different growth media as described above, or with different genetic inventories. We could therefore successfully apply whole-cell analysis to analyze the metal content in mutant strains impacted in Ni or Mo homeostasis. We expected strong impacts of systemic mutations and lower, but nevertheless significant, changes

due to structural gene mutations encoding abundant metalloproteins. Indeed, the systemic knockout of Ni-transporter genes (Δ*nikC* or Δ*nikE*) strongly impacted intracellular Ni levels. Similarly, the knockout of the gene encoding the FNR protein, which is a global regulator necessary, e.g., for transcription of the Ni-transporter genes,[49] strongly reduced Ni levels in both tested *E. coli* cultivations. This confirms earlier descriptions that FNR is the primary regulator for Ni transport but also shows the power of whole-cell metallomics for the detection of systemic mutations. Even more pronounced was the reduction of Mo abundance with the systemic knockout of *moaA*. MoaA catalyzes the first step of molybdopterin biosynthesis[50] and forms the major route to incorporate Mo into *E. coli* proteins. The overall low Mo content demonstrates that Mo does not accumulate in the cells if it cannot be incorporated into its target enzymes. This suggests feedback regulation between transport and the cofactor biosynthetic pathway[51], however, we can also not exclude an increased efflux. Residual Mo might have been bound to the transporter, might be freely soluble in the cytoplasm as molybdate anions or might be unspecifically attached to other cell components.

Strains with knockouts of genes encoding abundant metalloproteins were diminished in their respective metal content, demonstrating that whole-cell metallomics can be applied to examine the metal content of single abundant proteins. The overall Ni content in *E. coli* was ~25% lower after the knockout of a [NiFe] hydrogenases (Hyd-1, 2 and 3), suggesting that ~25% of the total cellular Ni amount, corresponding to ~2000 ions, was bound to these hydrogenases.[36] The fact that the complementation with HybC restored original Ni levels reveals that the overproduced HybC contains Ni indicating correct maturation and confirming earlier enzymatic observations.[49] Moreover, it confirms that HybC is the Ni-containing subunit, and demonstrates that the Ni transport and storage system is closely linked to the incorporation of Ni into metalloproteins. Clearly, whole-cell metallomics can also be used to identify yet-unknown and abundant metalloproteins by screening mutants for metal content, as was proposed earlier.[1]

Finally, we applied whole-cell metallomics to try to answer the question if OmeA, a protein in the organohalide respiration complex of *D. mccartyi*[30, 52] with a Mo-*bis*-MGD-binding site, contains Mo cofactors when overexpressed in an *E. coli* host. The wild type again showed significantly higher Mo levels when grown with nitrate compared with when oxygen was the terminal electron acceptor, indicating that the Mo-containing formate dehydrogenase and nitrate reductase bind about two thirds of the total cellular Mo under these conditions. The *moaA* knockout strain reduced Mo content significantly, especially under nitrate-reducing conditions. After heterologous overproduction of the OmeAB heterodimer in this mutant strain we observed a small increase in Mo content, which however proved not to be significant. Despite several repetitions of this experiment, no clear increase of the Mo content was observed, although the apoprotein was present in large amounts. We must interpret this data that it indicates that the *E. coli* Mo-cofactor incorporation machinery was ineffective at incorporation of the Mo-cofactor into the heterologously produced protein. This could either be due to

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the fact that the heterologously expressed enzyme was misfolded, or that the correctly folded OmeA protein does not bind Mo, as has been described for other metalloproteins with a predicted Mo-*bis*-MGD binding site.[53, 54]

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Conclusion

ICP-QqQ-MS can be applied to obtain absolute quantification of the cellular content of biologically important metals in whole cells at very high sensitivity so that the metal content can be analyzed also from microorganisms for which only low amounts of cell mass is available. The meaningful interpretation of whole-cell metallomics data is possible if no metal precipitates are formed. While we have identified single metalloproteins that contribute a major share of the total amount of a metal within a cell, it would be worth analyzing in detail all contributions of different metalloproteins[1] to the total content of different metals to identify gaps of our knowledge. If further elaborated, the method could be a tool to quantify metalloproteins in a facile, rapid and reliable way. Whole-cell metallomics showed particular strength in tracing the effects of mutations involved in metal homeostasis on the cellular metal content. Whereas systemic mutations, e.g. in metal transport or metal-cluster assembly, are readily identifiable, the consequences of mutations in genes encoding metalloproteins are only detectable if these proteins are abundant and if the metal-binding property of the protein is affected. In summary, whole-cell metabolomics represents a very fast, easy and strictly quantitative approach that can give direct information about the metal homeostasis of a microbial cell.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

R.B. and L.A. conceived the study and designed the experiments in coordination with G.S. and S.W. The lab experiments were done by R.B., D.C. and P.W, data was analyzed by R.B. and L.A.. R.B. and L.A. wrote the manuscript, and G.S., D.C., S.W. and T.R. contributed to the interpretation of the data and edited the manuscript.

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Tables

Table 1 *E. coli* mutant strains used for metal analysis

No.	Mutant Name	Description
1	BW25113	Wild-type *E. coli* strain: K12 derivative, Δ*araBAD*, Δ*rhaBAD*[55]
2	MC4100	Wild-type *E. coli* strain F-, [araD139] (*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB5301 rspL150* [56]
3	JW3443 Δ*nikC*	BW25113 but Δ*nikC* Kan^R [57]
4	JW3445 Δ*nikE*	BW25113 but Δ*nikE* Kan^R [57]
5	JW1328 Δ*fnr*	BW25113 but Δ*fnr* Kan^R [57]
6	MC4100 Δ*moaA*	MC4100 but Δ*moaA* Kan^R (Kind gift from N. Dragomirova)
7	FTD147	MC4100 with deletion mutations in catalytic subunits of three [NiFe] hydrogenase subunits (Δ*hyaB*, Δ*hybC*, Δ*hycE*)[58]
8	FTD147 + pASK-hybC	FTD147 with pASK-hybC (Amp^R), which overproduces HybC, the large subunit of [NiFe] hydrogenase 2 [49]
9	FTD150 Δ*selB*	MC4100 with knockout of three hydrogenase subunits (Δ*hyaB*, Δ*hybC*, Δ*hycE*), Δ*hyfB-R* and the Δ*selB* gene, encoding the SelB translation factor[21]
10	FTD150 Δ*selB* + pomeAB[a]	FTD150 Δ*selB* with pomeAB (Cm^R), which overproduced the molybdoprotein subunit OmeA (Kind gift from S. Hartwig)

[a] The subunits of the originally named CISM enzyme from *D. mccartyi* have been recently renamed as OmeA and OmeB.[59]

Table 2 Isotopes selected for quantification of the target metals, their limit of detection (LOD) and background equivalent concentration (BEC).

Metal isotope	LOD (nM)	LOD (ng L^{-1})	BEC (nM)
^{56}Fe	2.68	150	17.41
^{59}Co	0.02	1	0.08
^{60}Ni	0.98	59	1.29
^{95}Mo	0.26	25	1.06

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Figures

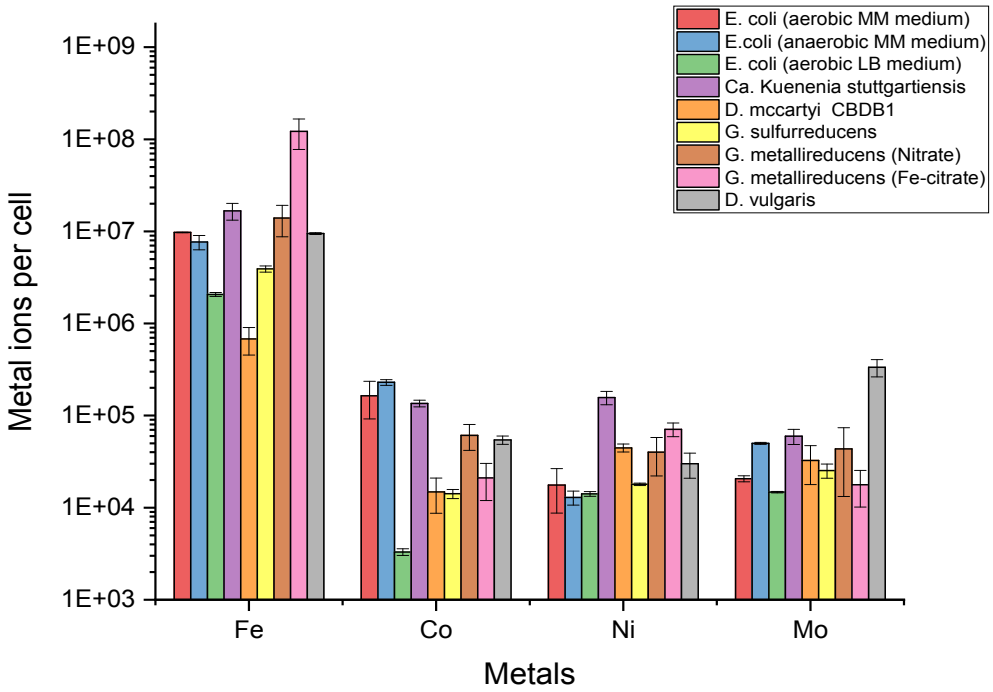

Fig. 1 Fe, Co, Ni and Mo content of whole cells of different bacteria as determined by ICP-MS. All cells were analyzed in three biological replicates and data are plotted as means ± SD. Each biological replicate was analyzed in three independent technical measurements and the mean was further used.

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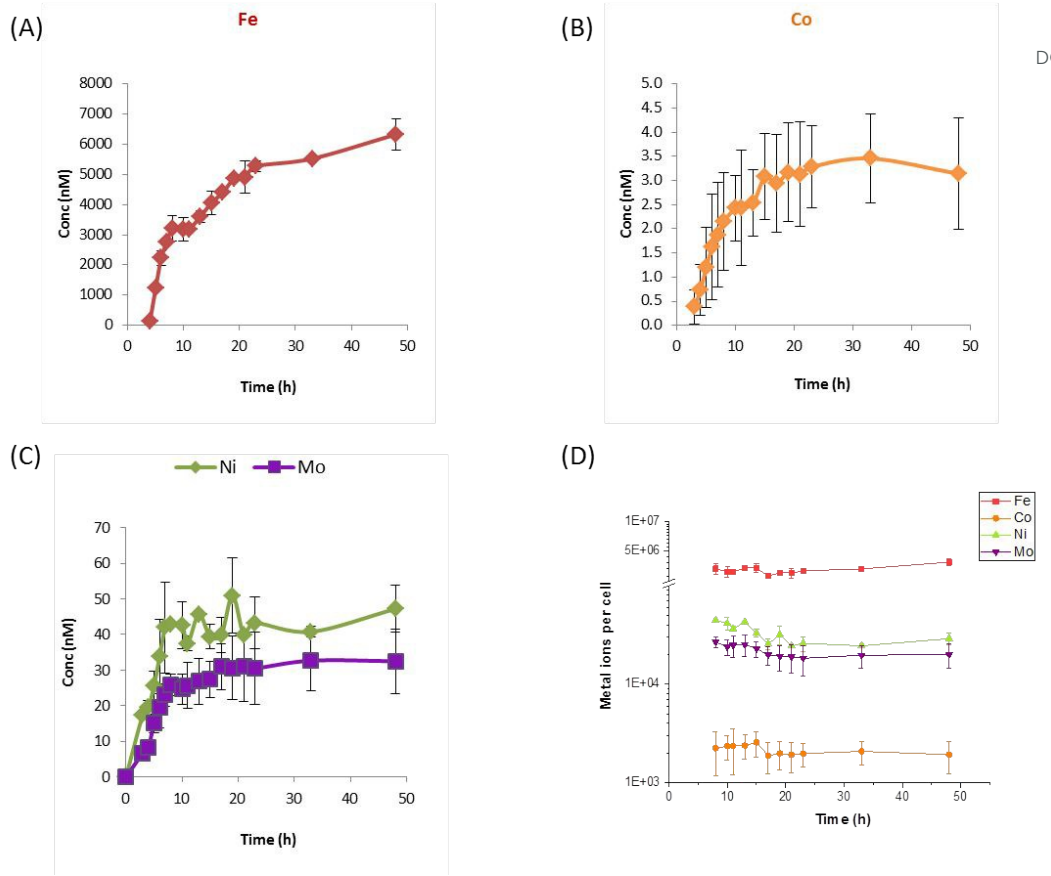

Fig. 2 Metal content of *E. coli* strain BW25113 after aerobic growth in LB medium for 48 h; (A-C) concentration of biomass-bound metals calculated to the culture volume at different time points; (D) same data as in A-C but data were related to the cell number in the culture to obtain absolute numbers of ions per cell. All values are means of three independent measurements ± SD.

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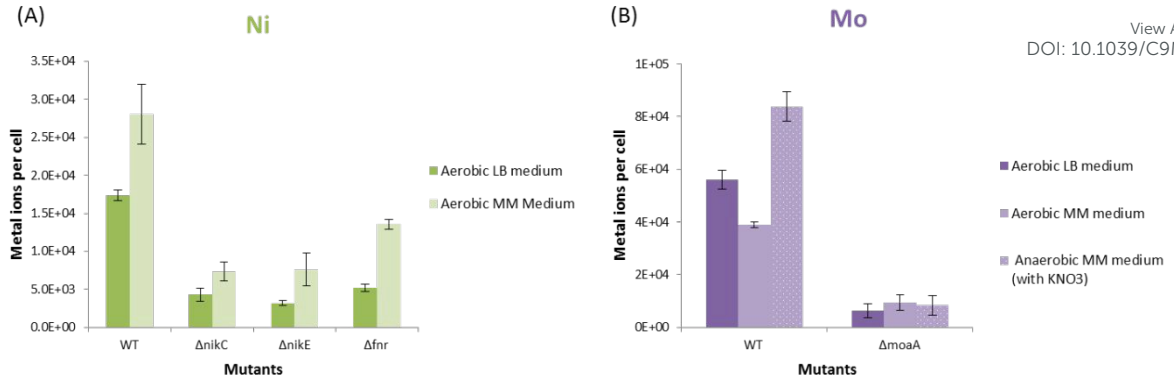

Fig. 3 Ni and Mo content in systemic *E. coli* knockout mutants. (A) Ni content in the *E. coli* wild-type strain BW25113 (WT) and in the mutants Δ*nikC*, Δ*nikE* and Δ*fnr*, grown aerobically in LB medium or aerobically in minimal medium (MM) A significant difference (p** < 0.01) was observed for all the mutant strains (Δ*nikC*, Δ*nikE* and Δ*fnr*) against wild-type strain. (B) Mo content in the wild-type strain BW25113 and in a Δ*moaA* mutant, grown aerobically in LB medium, aerobically in MM or anoxically in MM with KNO_3 as terminal electron acceptor. A significant difference (p** < 0.01) was observed for Δ*moaA* mutant against wild-type strain. The data show means of biological triplicates ± SD.

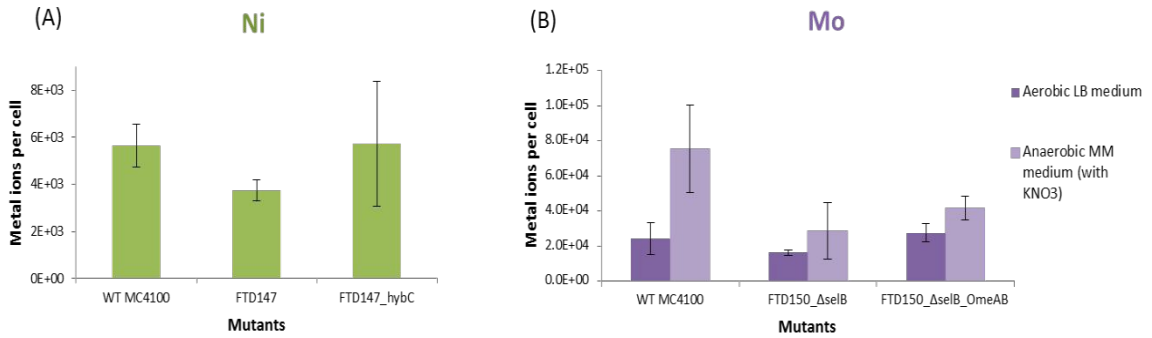

Fig. 4 Ni and Mo content in structural gene mutants. (A) Ni content in the wild-type strain MC4100, a mutant missing three subunits Δ*hyaB*, Δ*hybC*, Δ*hycE* of a [NiFe] hydrogenase (FTD147), and the same mutant FTD147 overexpressing *hybC* (FTD147_HybC). However, the difference between wild-type strain and FTD147 mutant (p = 0.07), and the difference between FTD147 mutant and

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FTD_hybC mutant (p = 0.06) was statistically not significant. (B) Mo content in the wild-type strain MC4100, in a strain mutated in the genes encoding the catalytic subunits of all four hydrogenases, and with a defect in synthesis of all three formate dehydrogenase genes due to deletion of *selB* (FTD150_Δ*selB*), and the same strain FTD150_Δ*selB* mutant heterologously overproducing OmeA and OmeB from *Dehalococcoides mccartyi* strain CBDB1.[30, 52] OmeA contains a molybdopterin binding-site. However, the difference between wild-type strain and FTD150_Δ*selB* mutant (p = 0.142), and the difference between FTD150_Δ*selB* mutant and FTD150_Δ*selB*_OmeAB mutant (p = 0.171) was statistically not significant. The data show means of biological triplicates ± SD.

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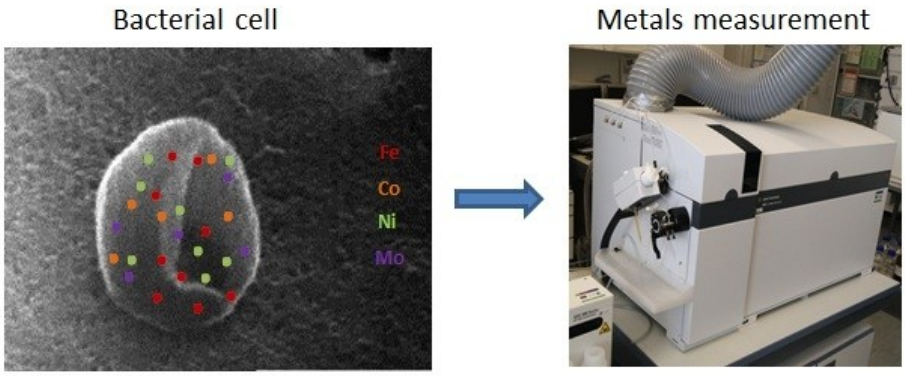

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